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Genetic susceptibility to toxicologic lung responses among inbred mouse strains following exposure to carbon nanotubes and profiling of underlying gene networks

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Abstract

The risk of human exposure to fiber nanoparticles has risen in recent years due to increases in the manufacture and utilization of carbon nanotubes (CNTs). CNTs are present as airborne particulates in occupational settings and their hazard potential has been demonstrated in experimental lung exposure studies using inbred mouse strains. However, it is not known whether different inbred strains differ in lung responses to CNTs by virtue of their genetics. In this work, common inbred strains (BALB/c, C57Bl/6, DBA/2, and C3H/He) were exposed to CNTs via oropharyngeal aspiration and lung histology and bronchoalveolar lavage (BAL) samples were evaluated over 28 days with the objective of evaluating sensitivity/resistance among strains. C57Bl/6 mice developed significantly more extensive type II pneumocyte (T2P) hyperplasia and alveolar infiltrate compared to DBA/2 mice, which were resistant. Surprisingly, DBA/2 but not C57Bl/6 mice were extremely sensitive to increases in leukocytes recovered in BAL fluid. Underlying global gene expression patterns in the two strains were compared using mRNA sequencing to investigate regulatory networks associated with the different effects. The impact of exposure on gene networks regulating various aspects of immune response and cell survival was limited in DBA/2 mice compared to C57Bl/6. Investigation of B6D2F1 (C57Bl/6 x DBA/2 hybrid) mice demonstrated inheritance of sensitivity to CNT exposures in regard to toxicologic lung pathology and BAL leukocyte accumulations. These findings demonstrate genetic bases of susceptibility to CNT particle exposures and both inform the use of inbred mouse models and suggest the likelihood of differences in genetic susceptibility among humans.

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Keywords

mouse models; inbred strains; genetic susceptibility; carbon nanotubes; nanoparticles; toxicologic pathology

Introduction

Environmental lung diseases resulting from exposure to biopersistent airborne particles continue to increase public health costs and affect quality of life among exposed populations (Maynard and Kuempel, 2005; Pope et al., 1995). Common aspects of particle-related lung disease include lengthy prodromal periods and low visibility, both of which increase the likelihood of advanced disease progression prior to any clinical detection and intervention. Prevention and monitoring are therefore important for controlling the public health impacts of environmental and occupational lung diseases and complications brought about by exposure to airborne particles. A significant component of this concerns inter-individual differences in susceptibility to exposure-related illness based on genetic variability. It is now appreciated that gene-environment interactions, in which one or many elements of genetic background modify toxicological responses to an exposure, play a critical and recurring role in the development of environmental disease and often underpin the observation of non-uniform outcomes among individuals with the same exposure status (Chamailard et al., 2003; Matsuo, 2001; Peden, 2005). The identification of genetic variants which determine sensitivity or resistance to the effects of environmental toxicants will play a significant role in identifying susceptible individuals among environmentally and occupationally exposed populations. This will aid in managing disease risk and limiting the impact and cost of particle-related diseases in the public sector.

Inbred mouse strains are a staple of biological and toxicological research. Because of the uniform genetic background between animals and subsequently low level of variability in responses to experimental exposures, surveys of inbred strains are a useful way to demonstrate the role of genetic variability in determining the outcome of environmental exposures. This has been demonstrated in cases of inhalational exposure to combustion particles (Kodavanti et al., 1997; Ohtsuka et al., 2000), metal oxides (Wesselkamper et al., 2001), diesel exhaust (Miyabara et al., 1998), carbon black (Hamade and Tankersley, 2009), ozone (Prows et al., 1997), and others. Genetic factors are thought and often shown to modify the risk of toxic responses of the respiratory system in animal models including chronic inflammation, fibrosis, airway hypersensitivity, cardiovascular complications, and carcinogenesis (Kleeberger, 2005; Peden, 2005). Concurrently, there are many studies which support roles for genetic factors in the risk of coal worker's pneumoconiosis, farmer's lung disease, silicosis, fibrosing alveolitis (idiopathic pulmonary fibrosis), asbestosis, and malignant mesothelioma (Hirvonen et al., 1995; Honda et al., 1993; Yucesoy and Luster, 2007). In particular, there has been interest in identifying genetic factors which may determine risk of malignant mesothelioma among asbestos-exposed human populations, however this has proven difficult to demonstrate due to large numbers of confounding factors (such as co-exposures) and small sample sizes (Horská et al., 2006; Neri et al., 2008).

Carbon nanoparticles have received attention in the environmental health and toxicology fields due to their persistence and potential to be inhaled as aerosols. In particular, carbon nanotubes (CNTs) are held to be important emerging toxicants as their manufacture and application continue to increase dramatically (Grand View Research, 2015.; De Volder et al., 2013). CNTs are high aspect ratio nanofibers which have been experimentally reported to cause chronic inflammation, hyperplasia, and fibrosis of lung tissue (Bonner et al., 2013; Frank et al., 2016; Mercer et al., 2011). Existing literature indicates that CNTs are poorly cleared from the lung in experimental models using inhaled or aspirated exposure doses (Elgrabli et al., 2008; Muller et al., 2005). The batch-to-batch material characteristics of CNTs vary and although progress continues to be made on the physicochemical determinants of toxicity, the biological mechanisms mediating toxic responses are not fully understood. Moreover, the extent to which genetic background may influence the qualities and severity of CNT-related toxic endpoints in the lung has not been reported. This is further relevant to the continuing toxicological investigations on CNTs, in which the use of inbred rodent strains is a critical platform for experimentation. The genetic homogeneity of a given strain may limit its ability to model exposures in humans as responses seen in a single inbred strain may be idiosyncratic of that strain (Montagutelli, 2000; Sundberg et al., 1991), thus differences in response to CNT exposures among strains should be investigated. In this work, we tested the hypothesis that genetic background plays a role in determining the nature and extent of adverse lung responses to carbon nanotube exposure. To test this, we performed a survey of inflammatory and pathological lung responses to experimental CNT exposure among inbred mouse strains with the goal of identifying sensitive and resistant strains. Doing this may demonstrate whether differences in genetic background may modify susceptibility to CNT-related lung complications and may also inform future investigations as to the implications of strain selection in toxicology studies. In addition, assessment of gene expression differences between sensitive and resistant strains may reveal clues as to what biological mechanisms determine susceptibility between individuals.

Methods

CNTs used in this study

Multi-walled CNTs were prepared, characterized, and used as previously described in Frank et al., 2015 (Frank et al., 2015). Briefly, physicochemical characterization of the CNT material indicated low average diameter (10.7 nm), tangled morphology resulting in loose agglomerates of fibers, negligible metal content, and no detectable endotoxin.

Animal Procedures

The protocols in this study were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Six week-old wild-type male C57Bl/6, BALB/c, C3H/He, DBA/2, and B6D2F1 mice were obtained from the Jackson Laboratory and were maintained and handled in the Laboratory Animal Maintenance Services facility at UC. Following a 1-week acclimation period, groups of all strains were exposed to CNTs dispersed in PBS with 1% Pluronic F127 or vehicle-only via orotracheal (oropharyngeal) aspiration as described previously (Frank et al., 2015). An exposure dose of 4 mg/kg body weight was chosen based on the results of a preliminary range finding experiment using

C57Bl/6 (see supplementary info, Fig. S1). Animals were sacrificed at 1, 14, or 28 d post-exposure ($n=5$ /group). Animals were euthanized with 20 μ L IP injections of Euthasol. Exsanguination was performed via transection of the abdominal artery. Lungs were perfused with 10mL PBS via the right ventricle. The left main bronchus was clamped and the trachea was exposed and cannulated for bronchoalveolar lavage (BAL). Right lungs were lavaged with 2 x 0.8 mL PBS and cells were retrieved from BAL fluids via centrifugation. After BAL of right lungs, the left lungs were unclamped and the clamp was moved to the right bronchus. Right lung lobes were removed and snap-frozen in liquid nitrogen. Left lungs were then inflated with formalin from a gravity stand at a height of 10 cm through the preexisting tracheal incision. Formalin-inflated left lungs were then removed and fixed in formalin for 24h. Lungs were then embedded in paraffin, sections, and stained with hematoxylin & eosin (H&E) at the Pathology Research Core at Cincinnati Children's Hospital Medical Center (CCHMC, Cincinnati, OH). In further analysis, selected samples were sectioned and stained with Masson's trichrome and immunohistochemical (IHC) stains for CD68, TTF-1, CD3, and CD31 markers using 3,3'-diaminobenzidine (DA B) chromogen and hemotoxylin counterstain for visualization.

Analysis of tissue histology

H&E- and Masson's trichrome-stained sections were evaluated for histopathology by a board-certified veterinary pathologist in a blinded format. For H&E stained sections, severity of histopathological changes were given scores of 0 (no change), 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked). Based on preliminary experiments, the following features were scored: granulomatous inflammation (i.e., cellular infiltrates composed predominantly of reactive macrophages), perivascular (P/ V) and interstitial (I/ S) lymphoid tissue hyperplasia, alveolar wall thickening, neutrophilic infiltration, foamy alveolar macrophages, type II pneumocyte (T2P) hyperplasia, and bronchoalveolar hyperplasia. Masson's trichrome stains were graded semiquantitatively as previously published (Izbicki et al., 2002) and were given scores of 0 (collagen content within normal limits), 1 (small focal areas of fibrosis), 2 (small multifocal areas), 3 (moderate focal areas of fibrosis), 4 (moderate multifocal areas of fibrosis), or 5 (extensive coalescing areas of fibrosis).

Analyses of BAL Fluids

Centrifuged cells retrieved from BAL fluids were resuspended in PBS with 5% FBS and counted via hemacytometer. Cells were then adsorbed to slides via cytospin and differential cell counts were performed based on morphology following Hema3 staining. Total protein in cell-free BAL fluids was measured separately using the Bio-Rad DC Total Protein Assay.

Statistical Analysis

Differences in ordinal scores for pathology were evaluated using a Mann-Whitney-Wilcoxon test. Differences in continuous data (cell counts and protein levels) were evaluated using a two-way ANOVA followed by Tukey post-hoc analysis. All statistical tests (apart from those used in differential gene expression analysis, described below) were performed in R v3.2.1 software. Data in figures are expressed as means $\pm$ SE, and statistical significance was noted were $p<0.05(*)$ or $p<0.01(**)$.

RNA Sequencing and Analysis

Sequencing of mRNA from vehicle - or CNT-exposed C57Bl/6 and DBA/2 lungs ($n=3$) was performed by the University of Cincinnati's Genomics, Epigenomics, and Sequencing Core. Briefly, polyA RNA isolation was carried out using WaferGen Apollo 324 system and PrepX PolyA script. The isolated RNA was RNaseIII-fragmented and Superscript III reverse transcriptase (Lifetech) was used to generate cDNA which was purified using Agencourt AMPure XP beads (Beckman Coulter). Individually indexed libraries were proportionally pooled (30 to 50 million reads) for clustering in cBot system (Illumina). Libraries were clustered onto a flow cell using Illumina's TruSeq SR Cluster kit v 3 and sequenced for 50 cycles using TruSeq SBS kit on the Illumina HiSeq system. The Sequence reads from the RNA-seq analysis were initially processed and exported using standard Illumina sequence analysis tools. Sequence reads were then aligned to the reference genome using the TopHat aligner (Trapnell et al., 2009), and reads aligning to each known transcript were counted using Bioconductor packages for next-generation sequencing data analysis (Huber et al., 2015). The differential expression analysis between different sample types was performed using the negative binomial statistical model of read counts as implemented in the edgeR Bioconductor package (Anders et al., 2013). The statistical significance of differential expression is established based on the FDR-adjusted p-values (Storey and Tibshirani, 2003). Differentially expressed gene networks were interpreted using Ingenuity Pathway Analysis (IPA) software (Qiagen).

Results

Pathophysiological differences among strains

No overt morbidity was observed in any group during the duration of the exposures and all but one animal survived until scheduled necropsy. One DBA/2 mouse which had been exposed to CNTs was discovered deceased but this did not appear to be related to any experimental variables. Cardiac calcinosis of the right ventricle was also seen in the majority of DBA/2 mice across the experiment, and is a common background lesion in this strain (Van Vleet and Ferrans, 1987).

In H&E-stained lung sections, no departure from normal tissue microstructure was apparent in vehicle-treated (VH) groups in any time point apart from the mild prevalence of foamy macrophages, which may be attributed to administration of the surfactant vehicle. Foamy macrophages were, overall, slightly more prevalent in C57Bl/6 compared to others strains in both vehicle and CNT-exposed groups, particularly at 1 and 28 d post-exposure. In 1 d CNT-exposed groups, histopathology showed limited T2P hyperplasia and modest neutrophilic infiltration was evident but no notable differences between strains were apparent at this time point (see supplementary information, Fig. S2). BAL fluid analysis showed increases in polymorphonuclear neutrophils brought on by CNT exposure with concurrent increases in total protein in cell-free fractions. Neutrophilia and total protein increases did not differ significantly between strains. Numbers of macrophages in CNT-exposed samples did not change significantly but were marginally higher in C57Bl/6 compared to other strains in both VH- and CNT-exposed samples.

At 14 days post-exposure, granulomatous inflammation was evident to some extent in all CNT-exposed strains, and was the most extensive in C57Bl/6 and DBA/2 (Fig. 1). Mild perivascular lymphoid hyperplasia and alveolar wall thickening were also noted in exposed C57Bl/6, BALB/c, and C3H/He at this time point, but not DBA/2. Moderate type II pneumocyte hyperplasia was observed in CNT-exposed groups and was variable between strains, BALB/c showing the greatest severity and C3H/He showing the least. Taken together, C57Bl/6 showed the most extensive pathology by a small margin, while DBA/2 showed the least. However, differences between CNT-exposed strains remained limited at this duration of exposure. Analysis of BAL fluids (Fig. 2) showed increases in both macrophage and neutrophil cells in all CNT-exposed groups compared to vehicle. These increases were comparable among strains with the exception that DBA/2 mice showed substantially greater increases of both cell types, although total protein did not reflect this strain-specific difference.

At 28 days post-exposure, the extent of granulomatous inflammation in CNT-exposed groups ranged from minimal to mild and was consistent between strains (Fig. 3). Both interstitial and perivascular forms of lymphoid hyperplasia as well as alveolar wall thickening were evident in CNT-exposed groups at this time point and were greatest in C57Bl/6, while being inconspicuous in DBA/2. Neutrophilic infiltration was evident in the interstitium and alveoli of CNT-exposed C57Bl/6, but was not observed in other strains. Foamy macrophages were prevalent, to the greatest extent in C57Bl/6. Type II pneumocyte and bronchoalveolar hyperplasia were also present to the greatest extent in C57Bl/6, but only observed to be mild in DBA/2. Overall, C57Bl/6 showed consistently more severe histopathological changes after 28 d CNT exposures while DBA/2 showed the least severe (Fig. 4). Differences in perivascular lymphoid hyperplasia and foamy macrophages were statistically significant ($p < 0.05$) at this stage of the study ($n = 5$). It was furthermore observed that nodular alveolar foci developed in both strains following exposure (Fig. 5). BAL fluid analysis (Fig. 6) showed that the numbers of macrophages and neutrophils in C57Bl/6, BALB/c, and C3H/He strains had subsided to some degree from the levels seen at the 14 d time point. However, numbers of neutrophils remained elevated in CNT-exposed DBA/2 mice (Fig. 5a) and macrophages in 28 d CNT-exposed DBA/2 increased substantially from their 14 d levels. Total protein in BAL fluids reflected this difference and was considerably greater in CNT-exposed DBA/2 mice compared to the remaining strains (Fig. 5b).

Focused comparison of C57Bl/6 and DBA/2 strains

Lung fibrosis differences—Semiquantitative assessment of Masson's trichrome stains of CNT-exposed lungs from C57Bl/6 and DBA/2 mice was done to investigate strain-specific differences in the development of fibrosis. In general, CNT-exposed lungs of either strain exhibited small focal areas of fibrosis (scores of 1). One exposed C57Bl/6 sample showed small multifocal areas of fibrosis, but the fibrotic response in both strains was limited overall and was neither significant nor apparent (data not shown).

Cellular composition of nodular alveolar foci—Small, nodular alveolar foci developed in the lung of both C57Bl/6 and DBA/2 strains following 28-day CNT exposure. We sought to investigate the cellular composition of these foci to assess their relevance in

the context of the inter-strain differences in outcomes. Immunohistochemistry (IHC) was used to identify macrophages, epithelial cells, endothelial cells, and T-cells by CD68, TTF-1, CD31, and CD3 markers, respectively. Upon qualitative assessment, we observed that these cellular formations were composed of epithelial cells (TTF-1⁺) and T-cells (CD3⁺) mixed in with CD68⁺ macrophages (Fig. 7). Endothelial cells were generally confined to alveolar walls. The cellular composition of the foci did not appear to differ between C57Bl/6 and DBA/2 strains. (Note: Representative images in Fig. 7 were taken from less severely infiltrated lung regions in order to clearly visualize alveolar foci. Images in Fig. 7 may therefore convey less infiltration than those shown in Figs. 4 & 5.)

C57Bl/6 x DBA/2 F1 Hybrids

We investigated the susceptibility of 6-week-old male F1 hybrids (B6D2F1) generated from C57Bl/6 and DBA/2 parent strains to pathological and inflammatory lung responses to CNT exposures at 14 and 28 d post-exposure ($n=10$). This was done to provide insight as to how the susceptibility traits underpinning sensitivity/resistance may be inherited. In addition to evaluation of CNT-exposed F1 hybrids relative to the parent strains, additional samples of the CNT-exposed parent strains were generated to ensure batch-to-batch consistency. As a result, $n=7$ for CNT-exposed C57Bl/6 and DBA/2 groups in this phase of the study.

Tissue histopathology at 14 d post-exposure showed variable levels of lesion extent/severity in CNT-exposed F1 mice relative to the C57Bl/6 and DBA/2 parents (Fig. 8). The expected features of granulomatous inflammation, lymphoid hyperplasia, and foamy macrophages were seen, as well as type II pneumocyte and bronchoalveolar forms of hyperplasia. The range of pathology scores in F1 mice variably fell within or slightly greater or lesser than the mean scores in CNT-exposed parent groups. As expected, parent groups did not exhibit great differences in lung pathology at this time point and C57Bl/6 showed marginally greater overall pathology than DBA/2 as reflected in the sum-of-scores comparison. At 28 days post-exposure, differences in CNT-exposed parent strains were more pronounced and the F1 mice showed highly variable intermediate sensitivity to CNT-induced lung pathology relative to the sensitive/resistant parent strains, as reflected by the total pathology scores shown in Fig. 8. Fully expanded endpoint data on the histological analysis of the F1 mice (Fig. S3) is given in supplementary information. At this stage of the analysis, all differences in individual lung pathology endpoints between 28 d CNT-exposed C57Bl/6 and DBA/2 groups were statistically significant ($p<0.05$, $n=7$) with the exception of granulomatous inflammation, in which significant differences were not observed (Fig. 9).

With regard to BAL fluid cytology, vehicle-treated F1 mice did not exhibit any signs of leukocytic influx. BAL leukocyte increases in CNT-exposed C57Bl/6 and DBA/2 parent groups ($n=7$) showed polar responses at 14d and 28d time points as anticipated, with C57Bl/6 showing notably lower numbers of macrophages and neutrophils which declined between 14 and 28 days. DBA/2 mice again showed markedly higher numbers of neutrophils and macrophages, and these numbers increased between 14 and 28 days particularly in regard to macrophages. F1 animals showed intermediate responses at both time points, which were varied and all fell in the range observed between the parent strains. While the level of neutrophils in F1 mice remained consistent on average between 14 and 28 days, the number

of macrophages in F1 mice increased between 14 and 28 days in a manner reminiscent of the DBA/2 parent. On the whole, F1 animals exhibited variable intermediate sensitivity to CNT exposures relative to C57Bl/6 and DBA/2 parent strains, with the profile of macrophage recruitment between 14 and 28 days more resembling that of the DBA/2 parent group.

Gene expression profiling and differentially impacted gene networks

Lung tissue from C57Bl/6 and DBA/2 strains exposed to CNTs for 28 d ($n=3$) exhibited increased transcription of many genes. These increases were determined by comparing gene expression intensities in CNT-exposed lungs relative to vehicle-treated lungs respective of either strain. On overview, the higher sensitivity of C57Bl/6 mice to CNT-induced lung changes compared to DBA/2 was reflected in the respective magnitudes of statistically significant changes observed across the transcriptome, with CNT exposures inducing 6,026 differentially regulated genes in C57Bl/6 versus only 159 genes being significantly impacted in DBA/2 ($FDR<0.1$). We expect that many low-magnitude expression changes were not captured in CNT-exposed DBA/2 mice due to those changes not reaching statistical significance. The sequencing data discussed in this report was submitted to NCBI's Gene Expression Omnibus database and may be accessed under GEO Series accession number GSE86339 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86339>).

The biological significance of global gene expression changes were interpreted using IPA core analyses of CNT-exposed over vehicle-treated expression levels. The differential expression of genes as used in these analyses is expressed as the base 2 log value of the ratio of the expression intensities (abbreviated as exp log ratio or ELR in figures and tables) in the CNT-exposed group over that of vehicle-treated. Cutoffs for gene changes analyzed were set at exp log ratio = ± 1 and $p<0.01$. Core analyses run in this way on C57Bl/6 and DBA/2 groups were then compared directly (using IPA's comparison analysis function) to juxtapose canonical pathways and gene networks impacted by CNT exposure in either strain. Our analyses made use of IPA's 'activation z-score', a statistical measure of the match between the predicted relationship direction and the experimentally observed expression changes of genes within relevant networks (Kramer et al., 2014). Canonical pathways impacted by CNT exposure in C57Bl/6 vs. DBA/2 strains included NF- κ B signaling, TREM1 signaling, and p38 MAPK signaling (Table 1). Specific pathways predicted by IPA to be affected by CNT exposure were similar in both sensitive and resistant strains, but the impact was more extensive in the sensitive C57Bl/6 strain. Throughout the analysis it was evident that genes related to lung damage and inflammation were more extensively induced by CNT exposure in C57Bl/6 compared to DBA/2. Overall, the inter-strain differences in impact on canonical pathways as predicted by IPA appear to be reflective of the large difference in the number of differentially expressed genes following CNT exposure, with C57Bl/6 mice exhibiting changes in far more genes over the global profile.

Several gene networks recognized by IPA were impacted by CNT exposure in sensitive C57Bl/6 mice. To help elucidate signaling networks which may underpin sensitivity based on immunological reactivity, we merged the two highest-scoring networks, which were clustered around inflammatory molecules IL-1 β and TNF, respectively. This merged

network was then filtered for molecules related to the IPA ‘respiratory disease’ function (Fig. 10). Molecules in this filtered network, which include IL-33, TREM1, CCL17, CCL24, CCL28, CXCL13, CLEC4E, HA VCR2, RC3H1, and others, may warrant further assessment in mechanistic investigation of CNT-induced lung changes. This network was overlaid with the ‘lung cancer’ disease function, indicating several hits within this selective network which suggest that sensitivity to CNT exposure in the current model may extend to cancer promotion by CNTs under certain circumstances (such as co-exposure to a primary carcinogen, as has been demonstrated (Sargent et al., 2014)).

Using IPA, we ran another core analysis in which expression levels in C57Bl/6 were directly compared to those of DBA/2 to assess whether normalized baseline (vehicle-treated) differences in gene expression appear consistent with the more extensive pathological changes to CNT exposures in C57Bl/6 mice (or leukocyte accumulation in DBA/2). Based on normalized gene transcript levels in either strain, baseline gene signatures were consistent with inhibition of pro-inflammatory upstream regulators in DBA/2 lung relative to C57Bl/6 (Table 2). Upstream regulators predicted to be inhibited in DBA/2 mice included IL-1s, TNF, INF γ , and IL-12. This analysis suggests that although neither strain presents apparent background inflammation at baseline, C57Bl/6 mice maintain an elevated state of pro-inflammatory signaling compared to DBA/2. The particular regulators identified are also consistent with the known bias towards T_H1-mediated immune responses often observed in C57Bl/6 (Sellers et al., 2012), however in this study we were not able to specifically assess the intersection between this bias and susceptibility to lung changes following exposure.

Discussion

In this study, we comprehensively evaluated lung responses to CNTs in four inbred mouse strains and an F1 cohort generated from the polarized DBA/2 and C57Bl.6 strains. In assessing tissue histopathology in H&E-stained lung sections, we found that genetic variability between strains played a role in determining the severity of various CNT-induced pathological alterations including development of interstitial and perivascular lymphoid hyperplasia, alveolar wall thickening, neutrophilic infiltration, and type II pneumocyte and bronchoalveolar forms of hyperplasia. Of the four inbred strains initially tested, C57Bl/6 mice were the most sensitive in these changes while DBA/2 mice were relatively resistant. Leukocyte and total protein accumulations in BAL fluid also varied dependent on genetic background, as DBA/2 mice showed dramatic increases in BAL leukocytes and protein following CNT exposure compared to the other strains.

Neither C57Bl/6 nor DBA/2 mice demonstrated significant collagen deposition (fibrosis) in lung tissue, despite the robust exposure dose. This may have been partially due to the use of Pluronic F127 surfactant to stably disperse the CNTs. Another study (Wang et al., 2012) documented a decrease in the ability of CNTs to elicit TGF- β *in vivo* when using a related surfactant (Pluronic F108) as a dispersant. While there has been increasing adoption of BSA/phosphocholine dispersants in CNT aspiration studies, we opted to use Pluronic in this instance in the interest of contiguity with our previously published work (Frank et al., 2015, 2016) using this material. Although we find these dispersion and aspiration methods adequate to illustrate the comparisons captured in the current study, it should be noted that

attempts to accurately model human exposures (i.e., for risk analysis) should use true inhalation exposures appropriately adjusted for interspecies differences in respiratory tract dosimetry. In another note, this study was restricted to male mice. This, in part, helped to limit the study to a feasible size and the use of male animals is thought to reflect what is expected to be a predominantly male worker population considered to be at-risk of high-level CNT exposures in industrial settings. However, the diversifying workforce and demands of human health modeling increasingly emphasize the need to draw toxicity information from both genders and this is a limitation of the current study.

In male B6D2F1 first-generation hybrids of the polarized parent strains, variable intermediate sensitivities were seen in all histological and cytological endpoints. As both parent strains are assumed to be genetically homozygous, all F1 offspring should be identically heterozygous throughout the genome. If consistent levels of sensitivity were observed between individual F1 offspring, we may then infer that a low number of genetic loci determine sensitivity/resistance in the parent strains. Instead, we found that sensitivity varies widely between F1 animals. Holding the assumption that F1 hybrids from homozygous inbred parents will be identically heterozygous, we may infer that the variability in sensitivity seen here arises from biological variability accumulated over many newly-heterozygous genetic loci contributing to sensitivity/resistance. Thus, these findings support the assumption that overall susceptibility, including the differential susceptibilities to several measured lung pathological and cytological responses to CNT exposures, is complex and represents the outcome of several interrelated multigenic traits. Although more detailed analysis of quantitative trait localization using back-cross (N2) or inter-cross (F2) generations was outside the scope of this study, this aspect of the work sets the stage for further investigation into the identification of specific genetic loci determining sensitivity/resistance to CNT exposures using recombinant offspring.

C57Bl/6 and DBA/2 strains consistently displayed polarized profiles of toxicological response to CNT exposure in this study, where C57Bl/6 developed chronic inflammatory and hyperplastic lung lesions, while DBA/2 were resistant to these yet sensitive to leukocyte and protein accumulations in BAL fluid. Neutrophilic infiltration was histologically apparent in CNT-exposed C57Bl/6 mice, although far greater numbers of polymorphonuclear leukocytes were observed in the BAL fluids of DBA/2 mice that did not show infiltration of neutrophils at the tissue level. Intuitively, we would assume that histopathology should visualize this difference. Tracheobronchial airways were not collected in this study, so leukocytes present in BAL fluid could possibly have been recovered from tissues beyond the scope of lung sections. The discordance between BAL fluid and histopathology trends was further surprising given that inflammatory lesions in the lung are often expected to correlate with inflammatory leukocyte and protein increases as measured in BAL fluid. However, well-performed strain survey exposure studies (Wesselkamper et al., 2000, 2001) have demonstrated that discordance between BAL leukocytes, BAL protein, and toxicologic lung pathology may occur, particularly as a strain-specific trait. Unusually sensitive BAL leukocyte or protein increases in response to inhaled pollutants were documented (Wesselkamper et al., 2001) in DBA/2 mice and DBA/2 F1 offspring specifically, and these effects were dependent on the specific toxicants (e.g., zinc oxide, ozone, endotoxin, etc.) and exposure conditions. In the current study, DBA/2 mice exhibit strain-specific resistance to

pathologic lung injury and marked sensitivity to leukocyte accumulations as heritable traits. Our follow-up experiment in F1 hybrids suggests that these effects are not linked (i.e., sensitivity in BAL responses does not appear to correlate with resistance to pathological changes). Overall, it should be observed that inbred strains can yield highly idiosyncratic outcomes in toxicity studies insofar as inbred strains are meant to model toxicological responses in human populations. The study supports the possibility that separate responses (lung pathology, BAL cells, BAL protein) may be modified independently by genetic background and perhaps other variables.

As noted, other studies (Di Bonaventura et al., 2010; Wesselkamper et al., 2001; Wilson et al., 2007) have occasionally reported highly sensitive BAL leukocyte increases in experimental exposures in DBA/2 mice, but the mechanism underlying this is not clear. These effects could be related to c5 complement deficiency intrinsic to the DBA/2 strain (Radovanovic et al., 2011). The responsiveness of the aryl hydrocarbon receptor pathway is another well-known aspect in which the C57Bl/6 and DBA/2 strains exhibit polarity (Chang et al., 1993). In addition, it is known that these strains differ in their bias toward T-lymphocyte-mediated responses (Sellers et al., 2012), with C57Bl/6 biased toward T_H1-directed responses while DBA/2 is normally biased toward T_H2. Our findings regarding C57Bl/6 in this study are consistent with those in previously published studies of CNT exposure (Bonner et al., 2013; Girtsman et al., 2014; Wang et al., 2011), while the experimental exposure of DBA/2 mice to CNTs has not been reported. It should be noted that the CNTs used in this study took the form of small, tangled agglomerates as opposed to more rigid, higher-diameter fibers featured in some other studies (Mercer et al., 2011; Sargent et al., 2014), which may underpin differences in dose-based outcomes. It has been argued that thin-diameter CNTs which form tangled agglomerates act through a mode-of-action dependent on surface area and more typical of poorly-soluble fine particles as opposed to fiber characteristics (Pauluhn, 2014).

Analysis of global transcriptome changes following CNT exposure in sensitive/resistant mice showed that sensitivity to CNT-induced pathology correlated with impacts on canonical pathways and gene networks related to inflammation, lung damage, immune cell recruitment, and cell survival. Many of the genes impacted by exposure were also related to cancer and the development of tumors. It is evident from the comparative gene expression changes as well as direct comparison of gene expression in C57Bl/6 and DBA/2 that C57Bl/6 lung tissues are characterized by more extensive pro-inflammatory signaling both at baseline and upon being exposed to CNTs. This was reflected in the variety and extent of predicted activation of IPA canonical pathways in CNT-exposed C57Bl/6 mice, which included NF- κ B signaling, p38 MAPK signaling, dendritic cell maturation, TLR signaling, and TREM1 signaling (Table 1). Of these, the TREM1 pathway appeared to be of particular interest due to its strong activation in C57Bl/6 but absence thereof in DBA/2. TREM1 is a recently-discovered immunotyrosine receptor first found in myeloid cells and has been shown to be a potent accessory molecule regulating the extent of inflammation in response to infections (Bouchon et al., 2001). Its ligand(s) are unknown but TREM1 appears to mediate pro-inflammatory communication between immune cells. TREM1-deficient mice are shown to have effective lung infection clearance but reduced disease severity (Weber et al., 2014), suggesting that TREM1 signaling may be an important regulator of adverse

inflammatory pathology in the lung. In an *in vitro* toxicogenomic study of amorphous silica nanoparticles, cultured human T2Ps (A549 cell line) exhibited TREM1 pathway activation (using IPA) in response to exposure (Pisani et al., 2015). The expression of TREM 1 pathway activity in T2Ps could therefore plausibly be relevant to the T2P hyperplasia seen in susceptible mouse models of exposure to nanoparticles in the present study. Several other high-scoring immune-related canonical pathways (such as dendritic cell maturation and Toll-like receptor signaling) appear to be due to the same gene expression changes which underlie TREM1 activation, reflecting the overlap between these networks.

Observing that DBA/2 mice displayed inhibited pro-inflammatory signaling compared to C57Bl/6, we reviewed literature comparing infection and immunity in these strains. DBA/2 mice have been found to be susceptible to lung infections which include influenza (Trammell et al., 2012), *Mycobacterium tuberculosis* (Cardona et al., 2003), *Pseudomonas aeruginosa* (Gosselin et al., 1995), and *Bacillus anthracis* (Yadav et al., 2011). In models of hypersensitivity pneumonitis, C57Bl/6 mice are sensitive to lung disease while DBA/2 are resistant, and differential cytokine expression (chiefly IL-12) is shown to be the determining factor (Gudmundsson et al., 1998). Although no apparent differences in background inflammation exist between DBA/2 and C57Bl/6, the present data are suggestive that higher background transcription of downstream targets of Nf- κ B, IL-1 β , and the like is a significant underlying factor in sensitivity to lung injury from CNT exposures in mice. This hypothesis may be reflective of a more generalized adverse outcome pathway of toxic lung injury caused by poorly-cleared insoluble particles, especially if adverse lung pathology is assumed to be a function of clearance overload conditions (Oberdorster, 1995).

In closing, this work identified and profiled susceptibility to adverse lung changes following CNT exposure in common inbred mouse strains and is the first work to do so in our knowledge. Future work should continue to explore genetically determined differences in sensitivity to CNT exposures in a variety of model species with the goals of identifying susceptibility loci or alleles which have human analogues, and identifying key signaling events which may be utilized in the development of biomarkers and intervention strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Strain-specific effects followed bolus lung exposures to thin, tangled MWCNTS in mice
- Exposed C57Bl/6 exhibit more pronounced lung pathology and proinflammatory signaling
- Exposed DBA/2 respond with highly sensitive increases in leukocytes in lavage samples
- Genetic background is shown to play a role in nature and severity of adverse effects

Fig. 1. Limited differences in lung pathology were evident in 14 d CNT-exposed inbred mouse strains

A: Inflammatory lesions at this time point were generally not extensive, although small differences were noted between strains. DBA/2 mice did not develop lymphoid hyperplasia at this point. B: Type II pneumocyte hyperplasia was marginally more severe in BALB/c mice following CNT exposure.

Fig. 2. CNT-exposed DBA/2 mice exhibited markedly higher levels of neutrophils and macrophages at 14 d compared to other inbred strains

A : CNT exposures increased levels of macrophages and neutrophils in 14 d exposures, particularly so in DBA/2 mice. B: Levels of total protein in BAL fluids did not differ notably among CNT-exposed inbred strains, in contrast with DBA/2 mice showing increased cells relative to other strains. ** $p < 0.01$ (ANOVA/Tukey, differences among CNT-treated groups were evaluated).

Fig. 3. Lung inflammatory (A) and hyperplastic pathology (B) is most extensive in C57Bl/6 mice following 28 d exposure to CNTs

A: CNT-exposed C57Bl/6 mice developed more severe interstitial (I/S) and perivascular (P/V) lymphoid hyperplasia as well as wall thickening and neutrophilic infiltration compared to other strains. DBA/2 mice were the least affected. B: CNT-exposed C57Bl/6 mice exhibited the most severe type II pneumocyte and bronchoalveolar forms of hyperplasia compared to other strains. DBA/2 mice were resistant to developing hyperplasia following CNT exposure. $n=5$, $*p<0.05$ (Mann-Whitney-Wilcoxon, differences among CNT-treated groups were evaluated).

Fig. 4. Extent and distribution of lung pathology in 28 d CNT-exposed lung in C57Bl/6 and DBA/2 strains

H&E stain. A&B: Low magnification of CNT-exposed C57Bl/6 (A) and DBA/2 (B) lungs showing distribution of lesions. C&D: Mid-magnification of CNT-exposed C57Bl/6 (C) and DBA/2 (D) lungs showing extent and localization of diffuse alveolar pathology, which was more extensive in C57Bl/6.

Fig. 5. Alveolar lung pathology in CNT-exposed C57Bl/6 and DBA/2 lungs

H&E stain. Top row: Heavily infiltrated areas and alveolar occlusions appeared in C57Bl/6 (A) but not DBA/2 (B) lungs following CNT exposure. Bottom row: Nodular, alveolar cellular foci (arrows) appeared in both C57Bl/6 (C) and DBA/2 (D) lungs following CNT exposure

Fig. 6. CNT-exposed DBA/2 mice exhibited dramatically increased BAL leukocytes at 28d post-exposure compared to other strains

A: Neutrophils remain elevated and macrophages were greatly increased in CNT-exposed DBA/2 mice compared to other strains. B: Total protein in BAL fluid from DBA/2 mice correlated with the high numbers of BAL leukocytes at 28d post-exposure. ** $p < 0.01$ (ANOVA/Tukey, differences among CNT-treated groups were evaluated).

Fig. 7. Cell types composing alveolar cellular foci in CNT-exposed C57Bl/6 and DBA/2 mice
Immunohistochemical stains using DAB chromogen identified the following cell types (from top): macrophages by CD68 (A & B), epithelial cells by TTF-1 (nuclear, C & D), T-cells by CD3 (E & F), and endothelial cells by CD31 (G & H), in C57Bl/6 (A, C, E, G) and DBA/2 (B, D, F, H). Alveolar cellular foci (arrows) were observed to comprise primarily epithelial cells with occasional T-cells, and macrophages less frequently. These observations appeared to be consistent between CNT-exposed lungs of both mouse strains.

Fig. 8. B6D2 F1 hybrids showed variable intermediate sensitivity in lung responses to CNT exposure at 14 d (top) and 28 d (bottom)

Ten CNT-exposed F1 mice are shown (individually numbered 1-10) to express intermediate sensitivity relative to sensitive/resistant C57Bl/6 and DBA/2 mice ($n=7$ for parent strains). For F1 mice administered vehicle control (F1VH), $n=5$. Lung pathology is represented here as a sum of scores from all histopathology categories presented previously (Total Score). Enumeration of BAL cells (macrophage & neutrophil) in F1 mice showed features resembling those seen in the DBA/2 parent strain, with increases in macrophage cells seen between 14 and 28 d following CNT exposure.

Fig. 9. Lung pathology of DBA/2 mice compared to C57Bl/6 28 days following CNT exposure While granulomatous inflammation was comparable between strains, C57Bl/6 were significantly more sensitive than DBA/2 in the development of lymphoid hyperplasia (interstitial: I/S, and perivascular: P/ V), alveolar wall thickening, neutrophilic infiltration, foamy alveolar macrophages, and type II pneumocyte/bronchoalveolar hyperplasia. $n=7$, * $p<0.05$, ** $p<0.01$ (Mann-Whitney-Wilcoxon).

Fig. 10. Respiratory disease-related gene networks impacted by CNT exposure in sensitive C57Bl/6 mice

A: IPA networks containing the IL-1 β and TNF cytokines were merged and filtered for relevance to respiratory disease. Red represents upregulation following CNT exposure, green denotes downregulation. B: The network was overlaid with the lung cancer disease function in IPA, designating CNT-impacted genes found to be relevant to both respiratory disease and lung cancer.

Table 1

IPA Canonical Pathways induced by CNT exposure in C57Bl/6 and DBA/2 lungs. Values shown are activation z-scores.

Canonical Pathway	C57Bl/6, CNT/Vehicle	DBA/2, CNT/Vehicle
NF- κ B Signaling	4.31	1.63
TREM1 Signaling	4.80	N/A
p38 MAPK Signaling	3.21	1.34
Colorectal Cancer Metastasis Signaling	3.53	1.00
Dendritic Cell Maturation	3.90	N/A
Toll-like Receptor Signaling	2.89	1.00
PPAR Signaling	-2.67	-1.00
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.44	N/A
LXR/RXR Activation	-2.98	-0.38
IL-6 Signaling	2.84	0.45
Role of IL-17F in Allergic Inflammatory Airway Diseases	3.16	N/A
Melanocyte Development and Pigmentation Signaling	3.00	0.00
Leukocyte Extravasation Signaling	1.81	1.00
HMGB1 Signaling	2.67	N/A
cAMP-mediated signaling	2.56	N/A
Acute Phase Response		
Signaling	2.52	0.00
Oncostatin M Signaling	2.45	N/A
GM-CS F Signaling	2.33	0.00
B Cell Receptor Signaling	2.18	N/A
CNTF Signaling	2.12	0.00
Interferon Signaling	2.12	0.00
Cholecystokinin/Gastrin- mediated Signaling	2.11	N/A
Cardiomyocyte Differentiation via BMP Receptors	-2.00	0.00
Fc γ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.94	0.00
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.94	0.00

Table 2

Baseline differences in activation status of upstream regulators in (vehicle-treated) DBA/2 vs. C57Bl/6 lungs indicate inhibited pro-inflammatory signaling in DBA/2 mice compared to C57Bl/6.

Upstream Regulator	Exp Log Ratio	Predicted Activation State (C57Bl/6 vs DBA/2)	Activation z-score	p-value of overlap
lipopolysaccharide		Inhibited	-4.790	1.89E-17
IL10RA	-0.930	Activated	3.142	7.22E-15
IL1B	-0.898	Inhibited	-2.498	9.20E-13
IL21		Inhibited	-2.238	5.20E-12
IFNG	-2.920	Inhibited	-4.294	9.89E-12
Ifnar		Inhibited	-2.344	8.66E-10
MYD88		Inhibited	-2.146	3.07E-09
TNF		Inhibited	-2.776	3.90E-09
poly rI:rC-RNA		Inhibited	-4.655	2.28E-08
phorbol myristate acetate		Inhibited	-2.131	2.64E-08
IL12 (complex)		Inhibited	-2.679	3.20E-08